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The Mechanism of Inhibition by Acidosis of Gluconeogenesis from Lactate in Rat Liver

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1. Gluconeogenesis from lactate or pyruvate was studied in perfused livers from starved rats at perfusate pH 7.4 or under conditions simulating uncompensated metabolic acidosis (perfusate pH 6.7–6.8). 2. In 'acidotic' perfusions gluconeogenesis and uptake of lactate or pyruvate were decreased. 3. Measurement of hepatic intermediate metabolites suggested that the effect of acidosis was exerted at a stage preceding phosphoenolpyruvate. 4. Total intracellular oxaloacetate concentration was significantly decreased in the acidotic livers perfused with lactate. 5. It is suggested that decreased gluconeogenesis in acidosis is due to substrate limitation of phosphoenolpyruvate carboxykinase. 6. The possible reasons for the fall in oxaloacetate concentration in acidotic livers are discussed; two of the more likely mechanisms are inhibition of the pyruvate carboxylase system and a change in the [malate]/[oxaloacetate] ratio due to the fall in intracellular pH.

Hems *et al.* (1966) showed that gluconeogenesis from lactate in the isolated perfused rat liver was markedly inhibited when the pH of the perfusate was less than 7.1. Subsequently Lloyd *et al.* (1973) showed that, under similar conditions of simulated metabolic acidosis in the perfused rat liver, lactate uptake was also inhibited and that this occurred when hepatic mean intracellular pH had fallen below 7.05. The possibility that this phenomenon could contribute to the development of clinical lactic-acidosis (in which arterial pH is frequently below 7.0) and also be related to the therapeutic effects of bicarbonate infusion in this condition has been discussed (Lloyd *et al.*, 1973; Cohen & Iles, 1975; Cohen & Woods, 1976).

The object of the present work was to determine the mechanism of inhibition by acidosis of hepatic gluconeogenesis from lactate.

Materials and Methods

Animals

Inbred Glaxo Wistar rats of weight 120–200g were starved for 48h before the experiments.

Chemicals

Reagents were obtained from the following sources: L(+)-lactic acid was from Sigma (London) Chemical Co., London S.W.6, U.K.; 5,5'-dimethyl-oxazolidine-2,4-dione was from Travenol Corp.; sodium pyruvate and inulin were from British Drug Houses, Poole, Dorset, U.K.; 2-oxoglutarate and L-aspartic acid were from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; malate dehydrogenase, 3-hydroxybutyrate dehydrogenase and lactate dehydrogenase (rabbit heart), enolase, pyruvate kinase, phosphoglycerate mutase, citrate synthase, aspartate aminotransferase, 2,3-diphosphoglycerate, oxaloacetic acid, ADP, NAD^+ and NADH were from Boehringer Corp. (London) Ltd., London W.5, U.K.; powdered bovine serum albumin (fraction V) was obtained from Armour Corp.; [1-^{14}C]acetyl-CoA (sp. radioactivity 58mCi/mmol), hydroxy[^{14}C]methyl-inulin (sp. radioactivity 12.6mCi/mmol) and ^3H_2O (sp. radioactivity approx. 100μCi/ml) were from The Radiochemical Centre, Amersham, Bucks., U.K., and 5,5'-[2-^{14}C]dimethyloxazolidine-2,4-dione (11 mCi/mmol) was from New England Nuclear Corp., Dreieichenhain, West Germany. All other reagents were of analytical grade, obtained from British Drug Houses.

Methods

Isolated perfused liver preparations were set up by using a modification (Cohen *et al.*, 1973) of the method and apparatus described by Exton & Park (1967). In some groups of experiments, uncompensated metabolic acidosis was simulated by decreasing the bicarbonate content of the perfusate while maintaining pCO_2 at the control value of approx. 4.93kPa. These experiments are hereafter referred to as acidotic.

To prepare the perfusion medium, 16g of bovine albumin (not delipidated) was dissolved in 100ml of standard Krebs bicarbonate buffer (Krebs & Henseleit, 1932), modified by the omission of some of the $NaHCO_3$ to give a final concentration of 5mM. The albumin was then dialysed for 48h against two changes of 2 litres of similarly modified buffer. It was then divided into two portions, to which either NaCl (acidotic experiments) or $NaHCO_3$ (control experiments) was added to render the solution nominally iso-osmotic to standard Krebs buffer. Expired human erythrocytes (4–5 weeks post donation) were washed three times with 154mM-NaCl and twice with either standard Krebs buffer (control experiments) or buffer in which 20mM-$NaHCO_3$ was replaced by a similar concentration of NaCl. Washed erythrocytes were then added to the albumin solution to give a packed cell volume of 0.15. When equilibrated in the perfusion apparatus at 37°C and at the perfusion pCO_2 of approx. 4.93 kPa, the pH of the control medium was usually 7.1–7.2, and was adjusted before perfusion to 7.36–7.44 by addition of $NaHCO_3$ solution suitably diluted in a further modified buffer constructed to prevent changes in osmolality and other electrolyte concentrations. The 'acidotic' medium when equilibrated under the same conditions was of pH6.7–6.9 and was used without further adjustment of pH. Either sodium lactate or sodium pyruvate was added to give initial concentrations of approx. 12 or 7.5mM respectively.

The perfusion rate was 7.0ml/min per 100g body wt. All perfusions lasted for 60min. When lactate was the substrate the medium was recirculated; the volume was initially approx. 150ml and was re-measured at the end of the experiment. When pyruvate was the substrate the medium was not recirculated, because in a recirculating system the very rapid uptake of pyruvate would have decreased the perfusate concentration of pyruvate below the value at which uptake was maximum, and in addition there would have been substantial accumulation of lactate. The rate of substrate uptake and metabolic output was measured over the 40–60min period in the recirculating perfusions by measuring the fall in concentration in the reservoir over that period, and in the non-recirculating experiments by measuring the arteriovenous concentration differences across the liver and the flow rate; results were expressed as μmol/min per 100g original animal weight.

At the end of the experiment the liver was clamped with aluminium tongs precooled in liquid N_2 and ground under liquid N_2, and the powder transferred to a preweighed tube, which was reweighed and stored under liquid N_2 until deproteinization with 0.6M-$HClO_4$.

In a separate group of perfusions in which lactate was the substrate at an initial concentration of 10–12mM, hepatic intracellular pH was measured at the end of the experiment by determining the distribution of the weak acid 5,5′-dimethyloxazolidine-2,4-dione (Waddell & Butler, 1959). For this purpose 5,5′-[^{14}C]dimethyloxazolidine-2,4-dione, hydroxy[^{14}C]-methyl-inulin and ^3H_2O were added to the perfusate at the beginning of the experiment. The amounts added, the quantities of carrier 5,5′-dimethyloxazolidine-2,4-dione and inulin, the method of handling tissues and the calculations have been previously described (Cohen *et al.*, 1971).

Chemical methods

Perfusate lactate, pyruvate and glucose were determined by autoanalyser adaptations of the methods of Hohorst *et al.* (1959) and Huggett & Nixon (1957). The following manual methods were used for tissue metabolites, by using neutralized $HClO_4$ extracts of frozen tissue powder: lactate, Hohorst *et al.* (1959); D-(−)-3-hydroxybutyrate, Williamson & Mellanby (1963); acetoacetate, Mellanby & Williamson (1963); malate, Hohorst (1963); pyruvate, phosphoenolpyruvate, 2- and 3-phosphoglycerate, Czok & Eckert (1963); and L-aspartate, Pfleiderer (1963).

Tissue oxaloacetate was measured by the method of Wieland & Löffler (1974). In this method citrate synthase is used to condense oxaloacetate with [^{14}C]-acetyl-CoA, and the resulting labelled citrate is isolated and counted for radioactivity as the silver salt, the sensitivity depending on the specific radioactivity of the acetyl-CoA. Recovery experiments were performed in two ways, firstly by adding oxaloacetate to the $HClO_4$ supernatant after deproteinization, and secondly by adding it to the powdered frozen tissue before homogenization. The varying amount of oxaloacetate added was always less than that expected to be found in the experimental tissues, and, in the livers used for assessment of recovery from frozen tissue, the excised tissues were allowed to remain anoxic for 5min before freeze-clamping to lower their oxaloacetate content. In four recovery experiments from $HClO_4$ supernatant, in which the amounts of oxaloacetate added were about 25% of that initially present, the recoveries were 97–109% of those expected. In three experiments in which oxaloacetate was added to 4–5g of frozen tissue powder (measured content of oxaloacetate 0.0095 μmol/g) recoveries of oxaloacetate added in amounts of 0.015, 0.030 and 0.045μmol were 123, 107 and 111% respectively. The samples of tissue homogenate used in these recovery experiments were similar to those used in the definitive experiments.

Perfusate pH and pCO_2 were measured by using a Radiometer BMS 3 blood-gas analyser (Radiometer, Copenhagen, Denmark), by using calibrating buffers of nominal pH6.84 and 7.38.

Calculations

For lactate and pyruvate, tissue concentrations were corrected for admixture with extracellular-fluid lactate and pyruvate, by assuming an extracellular space of 27% of the tissue water (Cohen *et al.*, 1971). In the studies of Lloyd *et al.* (1973) there was no difference in the fractional extracellular space after 1h in groups of livers perfused at pH7.36–7.45 or 6.71–6.9. For 3-hydroxybutyrate and acetoacetate, tissue concentrations were corrected on the basis of extracellular/intracellular concentration ratios established in a separate group of experiments in which livers were perfused for 60min under similar control or acidotic conditions to those obtaining in the main experiments (see the Results section). It was assumed that aspartate, oxaloacetate, malate, phosphoenolpyruvate, 2- and 3-phosphoglycerate were entirely intracellular.

Means are expressed ±1S.E.M. Standard two-tailed *t* tests were used for comparisons between groups and correlations assessed by calculating standard parametric correlation coefficients.

Results

Metabolite concentrations in control and acidotic livers

Table 1 shows the results of experiments in which lactate was the substrate. The values of perfusate lactate concentration, pH and pCO_2 were obtained from the 'arterial' side of the preparation at the end of the experiment. The lower perfusate concentration of lactate in the control group is due to the greater uptake of lactate compared with the acidotic group. Mean lactate uptake was decreased in the acidotic group by 76.5% and glucose output by 64.3%. Intracellular lactate and malate concentrations were elevated, that of pyruvate was unchanged, and those of phosphoenolpyruvate, 2- and 3-phosphoglycerate were significantly decreased in the acidotic livers. The intracellular [lactate]/[pyruvate] ratio was almost doubled, and for this reason the elevation of malate concentration in the acidotic group might well be attributable to an elevation of the [malate]/[oxaloacetate] ratio and need not reflect a rise in oxaloacetate concentration, which was not measured in the experiments summarized in Table 1.

Table 2 shows the results of non-recirculating perfusion experiments in which pyruvate was the substrate. In the acidotic group pyruvate uptake was decreased by 44.3%, glucose output by 46.9% and lactate output by 38.8%. Intracellular pyruvate concentration was elevated in the acidotic experiments, those of malate and 2-phosphoglycerate were unchanged and those of phosphoenolpyruvate and 3-phosphoglycerate were decreased, though the fall in both cases just failed to achieve significance at the 5% level.

Table 1. *Lactate uptake, glucose output, perfusate lactate, pH and pCO_2 and tissue metabolites in control and acidotic livers perfused with lactate as substrate*

Perfusions were carried out as described in the Materials and Methods section. The perfusate values are those measured in perfusate from the 'arterial' side of the preparation at the end of the perfusion (60 min). The lactate uptake and glucose output were measured over the 40–60 min period. *$P<0.05$ compared with control, **$P<0.01$. The values are means±1S.E.M.; numbers of observations are given in parentheses.

	Mean perfusate lactate concn. (mM)	Perfusate		Lactate uptake (μmol/min per 100g body wt.)	Glucose output (μmol/min per 100g body wt.)	Liver metabolite concentration (mmol/l of intracellular water)							Intracellular [lactate]/[pyruvate]
		pH	pCO_2 (kPa)			Lactate	Pyruvate	Malate	Phosphoenolpyruvate	2-Phosphoglycerate	3-Phosphoglycerate		
Control	8.28±0.71 (8)	7.39 ±0.011 (8)	5.54±0.10 (7)	5.14 ±0.76 (8)	2.41 ±0.41 (8)	4.17 ±0.22 (7)	0.59±0.03 (7)	0.32 ±0.021 (7)	0.20 ±0.019 (7)	0.090 ±0.008 (7)	0.46 ±0.058 (7)	7.18	±0.38 (7)
Acidotic	11.4* ±0.68 (8)	6.72** ±0.050 (8)	5.63±0.11 (7)	1.21** ±0.39 (8)	0.86** ±0.091 (8)	8.42** ±0.44 (7)	0.68±0.06 (7)	0.40* ±0.024 (7)	0.11* ±0.017 (7)	0.054** ±0.006 (7)	0.29* ±0.039 (7)	13.89**	±1.08 (7)

Table 2. *Pyruvate uptake, glucose and lactate output, perfusate pyruvate, lactate, pH and pCO_2 and tissue metabolites in control and acidotic livers perfused with pyruvate as substrate*

Perfusions were carried out as described in the Materials and Methods section. Metabolite uptake/output was estimated after 60 min of perfusion by arteriovenous differences. See legend to Table 1 for other details. †$0.05 < P < 0.1$; *$0.01 < P < 0.05$; ***$P < 0.001$. There were six experiments in each group.

	Mean perfusate pyruvate (mM)	Mean perfusate lactate (mM)	Perfusate		Pyruvate uptake (μmol/min per 100g rat)	Glucose output (μmol/min per 100 g rat)	Lactate output (μmol/min per 100 g rat)	Liver metabolite concentrations (mmol/litre of intracellular water)						Tissue [lactate]/ [pyruvate]
			pH	pCO_2 (kPa)				Lactate	Pyruvate	Malate	Phospho-enolpyruvate	2-Phospho-glycerate	3-Phospho-glycerate	
Control	7.56±0.41	1.14±0.23	7.42 ±0.018	4.71±0.11	19.4 ±1.75	4.31 ±0.79	9.62 ±1.06	2.78±0.21	2.15 ±0.14	0.52±0.032	1.32 ±0.097	0.27±0.013	4.14±0.40	1.28±0.18
Acidotic	7.22±0.42	0.96±0.12	6.72***±0.043	5.00±0.26	10.8***±1.72	2.29*±0.49	5.89*±0.86	3.59±0.54	3.65*±0.38	0.51±0.026	0.94†±0.14	0.27±0.034	3.09±0.034	1.08±0.21

Table 3. *Oxaloacetate and other tissue metabolites in control and acidotic livers perfused with lactate as substrate*

Perfusions were carried out as described in the Materials and Methods section. See legend to Table 1 for other details. There were eight experiments in the control group and seven in the acidotic group. *$0.01 < P < 0.05$; †$0.01 < P < 0.02$; ‡$0.001 < P < 0.01$; §$P < 0.001$.

	Mean perfusate lactate (mM)	Perfusate		Lactate uptake (μmol/min per 100 g rat)	Glucose output (μmol/min per 100 g rat)	Liver metabolite concentration in intracellular water							Intracellular ratios	
		pH	pCO_2 (kPa)			Lactate (mM)	Pyruvate (mM)	Malate (mM)	3-Hydroxy-butyrate (mM)	Aceto-acetate (mM)	Aspartate mM)	Oxalo-acetate (μM)	[Lactate]/[pyruvate]	[3-Hydroxy-butyrate]/[aceto-acetate]
Control	6.92±0.50	7.40±0.019	5.60±0.93	4.94 ±0.41	2.32 ±0.35	4.49 ±0.37	0.52±0.046	0.40±0.06	0.13 ±0.013	0.21 ±0.018	0.45 ±0.050	11.01 ±1.14	9.37±1.45	0.65±0.080
Acidotic	7.43±0.34	6.80±0.043	5.61±0.08	3.17*±0.63	1.13*±0.34	9.76§±0.91	0.50±0.038	0.45±0.07	0.23§±0.015	0.31‡±0.023	0.28*±0.045	6.72†±0.85	20.1§ ±2.03	0.74±0.042

The results of Tables 1 and 2 suggest that the effect of acidosis is exerted at a step in gluconeogenesis preceding phosphoenolpyruvate. The experiments in Table 3 were an attempt to localize the action of acidosis more precisely. Measurements of tissue concentrations of lactate, pyruvate, malate, 3-hydroxybutyrate, acetoacetate, oxaloacetate and aspartate were made. Lactate was again the substrate; lactate uptake was decreased in the acidotic group by 35.8% and glucose output by 51.3%. Intracellular oxaloacetate concentration was lowered by 39% and aspartate by 38%; as in the experiments of Table 1, intracellular [lactate]/[pyruvate] was approximately doubled, owing entirely to a raised lactate concentration.

The distribution of 3-hydroxybutyrate and acetoacetate between the perfusate and the extracellular fluid was established as indicated in the Materials and Methods section. The mean intracellular/extracellular concentration ratios of 3-hydroxybutyrate and acetoacetate in the control series ($n = 6$) were 1.56 ± 0.35 and 0.69 ± 0.08 ($n = 4$) respectively; in the acidotic series these ratios were 1.72 ± 0.28 ($n = 4$) and 1.40 ± 0.14 ($n = 5$). These ratios were used to calculate the concentrations of these metabolites in intracellular water by the same method as described for lactate and pyruvate. In the acidotic series intracellular 3-hydroxybutyrate and acetoacetate concentrations were elevated. There was a positive correlation ($r = 0.58$; $P < 0.05$, $n = 15$) between intracellular [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] when the data from control and acidotic groups were pooled; there was also a significant correlation of data for the control group taken alone ($P < 0.05$), but not in those for the acidotic group.

Intracellular pH in control and acidotic perfused livers

In a separate series, intracellular pH was calculated from 5,5′-[^{14}C]dimethyloxazolidine-2,4-dione distribution at the end of the experiment in three groups of perfusions in which the mean final perfusate pH values were 7.42 ± 0.014 ($n = 5$), 7.13 ± 0.027 ($n = 6$) and 6.82 ± 0.027 ($n = 7$) respectively; final perfusate lactate concentrations were 9.1 ± 1.7, 6.9 ± 0.9 and 9.0 ± 1.6 mM respectively and mean intracellular pH 7.26 ± 0.025, 7.16 ± 0.044 and 6.92 ± 0.084 respectively.

Discussion

The observations recorded in Tables 1 and 2 suggested that the effect of acidosis in inhibiting hepatic gluconeogenesis from lactate or pyruvate is exerted at some point before phosphoenolpyruvate. The finding that hepatocyte oxaloacetate concentration is lowered in the acidotic experiments permits some further interpretation. A detailed analysis would require partitioning of the measured oxaloacetate between the cytosolic and mitochondrial compartments. Though attempts have previously been made to calculate this distribution, assumptions which are difficult to substantiate are required with respect to either the distribution of malate or the proportion of oxaloacetate bound to mitochondrial enzymes (Sols & Marco, 1970). However, mitochondrial oxaloacetate concentrations are thought to be an order of magnitude or more smaller than cytosol concentrations (Williamson *et al.*, 1967; D. H. Williamson *et al.*, 1969; Williamson, 1969), and, because of the small fraction (approx. 10%) that the mitochondrial water contributes to the total cell water, it is probable that the lower total cell oxaloacetate concentration observed in the acidotic experiments reflects a similar lowering of cytosol oxaloacetate concentration.

It is therefore possible that the decrease in hepatic gluconeogenesis from lactate in the acidotic livers is due to substrate limitation, by the lowered cytosol oxaloacetate concentration, of the rate of conversion of oxaloacetate into phosphoenolpyruvate. This possibility depends on the observed cytosol oxaloacetate concentrations being near the K_m *in vivo* for phosphoenolpyruvate carboxykinase, which in the rat liver is entirely located within the cytosol. Estimates of K_m for phosphoenolpyruvate carboxykinase *in vitro* vary widely with the assay system used, and the values obtained have been collated by Pogson & Smith (1975). For the reaction in the direction of phosphoenolpyruvate synthesis, values of K_m of 1.5–5 μM (Walsh & Chen, 1971) and 25 μM (Ballard, 1970) have been obtained. If similar values are relevant *in vivo*, then the concentration of oxaloacetate measured in the present studies clearly could be responsible for determining the rate of gluconeogenesis. It may be noted that the percentage decreases in lactate uptake (35.8%), glucose output (51.3%) and oxaloacetate concentration (39%) in the present study were very similar. It is noteworthy that hepatic phosphoenolpyruvate carboxykinase activity has been shown to be unchanged in animals rendered acidotic by administration of NH_4Cl (Alleyne & Scullard, 1969; Kamm & Cahill, 1969; Longshaw *et al.*, 1972), in contrast with the increase in activity found in the kidneys of similarly treated animals.

The reasons for the decrease in oxaloacetate concentration in liver perfused with media of low pH remain to be considered. Theoretically there are many possible mechanisms for this effect, namely: (*a*) inhibition of lactate transport across the plasma membrane; (*b*) inhibition of pyruvate transport across the mitochondrial membrane; (*c*) inhibition of the pyruvate carboxylase system; (*d*) inhibition of

oxaloacetate–aspartate transamination; (*e*) inhibition of aspartate exit from the mitochondria; (*f*) alteration of redox ratios. Taking these possibilities in turn, the first seems excluded, because cell lactate concentration is raised in the acidotic livers. Inhibition of pyruvate entry is also unlikely, because the specific mitochondrial-membrane pyruvate transporter (Halestrap, 1975) appears to carry H^+ ions in with pyruvate, and pyruvate entry into the mitochondria might therefore be expected to be enhanced by lowering of cytosol pH. Inhibition of the pyruvate carboxylase system is a distinct possibility, since *in vitro* both the activity of pyruvate carboxylase itself and its activation by acetyl-CoA are markedly decreased by a fall in pH from 7.8 to 6.9 (Scrutton & Utter, 1967; Söling *et al.*, 1970). However, obvious difficulties arise from lack of knowledge of the pH at the intramitochondrial site of localization of pyruvate carboxylase. A decrease in intramitochondrial acetyl-CoA concentration is also possible.

Inhibition of transamination (possibility *d*) seems at first sight unlikely, because the metabolic-profile changes during acidosis when pyruvate is the substrate (Table 2) are rather similar to those when lactate is the substrate, and in perfused livers transport of oxaloacetate out of the mitochondrion when pyruvate is the substrate does not appear to require transamination (J. R. Williamson *et al.*, 1969; Anderson *et al.*, 1971). However, a consideration of the balance of reducing equivalents in cytosol and mitochondria in the experiments with pyruvate casts some doubt on this interpretation. Conversion of pyruvate into glucose requires 1 reducing equivalent, which is probably transferred as malate from the mitochondria to the cytosol (Walter *et al.*, 1966). However, Table 2 shows that 50% of the pyruvate removed by the liver is converted into lactate. This requires a further reducing equivalent, and for this reason 2 malate molecules may leave the mitochondria for every molecule of pyruvate converted into glucose. This process would rapidly deplete the mitochondria of C_4 dicarboxylic acids, unless the C_4 skeleton of malate was returned to the mitochondria. It is suggested that the extra malate reaching the cytosol is converted by oxidation and transamination into aspartate, which enters the mitochondria; the aspartate then transaminates with 2-oxoglutarate, also derived from the cytosol, to form oxaloacetate and glutamate, the latter being returned to the cytosol. This scheme maintains both the carbon and nitrogen balance of the two compartments. If this system occurs, transamination must be involved in gluconeogenesis from pyruvate, and inhibition of transamination by acidosis is thus a possible explanation of the decreased gluconeogenesis from both lactate and pyruvate seen under these conditions. The observed decrease in tissue aspartate (Table 3) does not resolve this issue, since the distribution of this metabolite between the mitochondrion and cytosol is unknown; it may merely reflect the fall in its immediate precursor, oxaloacetate. Anderson *et al.* (1971) found that amino-oxyacetic acid, which is a transaminase inhibitor, has no effect on gluconeogenesis in perfused rat liver from pyruvate (2mM) but markedly inhibited gluconeogenesis from lactate (8mM). However, in these studies, which were all in recirculating perfusions, lactate production from pyruvate was very much smaller than in the present series.

Table 2 shows that acidosis inhibits pyruvate uptake and output of glucose and lactate to a similar degree. The scheme outlined in the last paragraph indicates that any mechanism which ultimately decreased the amount of malate transferred out of the mitochondria could, by diminishing the supply of reducing equivalents to the cytosol, affect to an equal degree conversion into lactate and that of 1,3-diphosphoglycerate into glyceraldehyde 3-phosphate (and thus glucose production).

Finally, the possibility must be considered that the mechanism leading to the observed alteration in the intracellular [lactate]/[pyruvate] ratio could be responsible for the lowering of oxaloacetate concentration. In both the series of experiments reported in Tables 1 and 3, the intracellular [lactate]/[pyruvate] ratio was approximately doubled. This is consistent with the fall in intracellular pH of about 0.3 unit, without involving any change in the free [NADH]/[NAD^+] ratio (Cohen & Woods, 1976); a similar situation has been observed in rat brain when subject to various partial pressures of CO_2 *in vivo* (Folbergrova *et al.*, 1972). Intracellular pH calculated from the distribution of 5,5′-dimethyloxazolidine-2,4-dione gives a type of volume-weighted 'mean' intracellular pH (Robson *et al.*, 1968; Cohen & Iles, 1975); in view of the large volume of cytosol compared with the mitochondrial water, and the improbability (Cohen & Iles, 1975) of the gross pH gradient across mitochondrial membranes demonstrable in certain conditions *in vitro* (Addanki *et al.*, 1967) being present *in vivo*, it is likely that the intracellular pH changes demonstrated are principally determined by changes in cytosol pH. The possibility arises that the cytosol [malate]/[oxaloacetate] ratio is affected in the same sense as the [lactate]/[pyruvate] ratio by a fall in cytosol pH; the unchanged malate concentrations suggest that a rise in the [malate]/[oxaloacetate] ratio could be due to a lowering of cytosol oxaloacetate concentration. The positive correlation of the [3-hydroxybutyrate]/[acetoacetate] ratio with the [lactate]/[pyruvate] ratio could reflect changes in mitochondrial pH directionally similar to those in the cytosol, and mitochondrial oxaloacetate concentration might fall by the same type of mechanism suggested in this paragraph for cytosol oxaloacetate.

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